Activation of the sweet taste receptor, T1R3, by the artificial sweetener sucralose regulates the pulmonary endothelium

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ABSTRACT

A hallmark of acute respiratory distress syndrome (ARDS) is pulmonary vascular permeability. In these settings, loss of barrier integrity is mediated by cell-contact disassembly and actin-remodelling. Studies into molecular mechanisms responsible for improving microvascular barrier function are therefore vital in the development of therapeutic targets for reducing vascular permeability in ARDS. The sweet taste receptor, T1R3 is a GPCR, activated following exposure to sweet molecules, to trigger a gustducin-dependent signal cascade. In recent years, extraoral locations for T1R3 have been identified however, no studies have focused on T1R3 within the vasculature. We hypothesise that activation of T1R3, in the pulmonary vasculature, plays a role in regulating endothelial barrier function in settings of ARDS.

Our study demonstrated expression of T1R3 within the pulmonary vasculature, with a drop in expression levels following exposure to barrier disruptive agents. Exposure of lung microvascular endothelial cells to the intensely sweet molecule, sucralose, attenuated LPS- and thrombin-induced endothelial barrier dysfunction. Likewise, sucralose exposure attenuated bacteria-induced lung edema formation *in vivo*. Inhibition of sweet taste signalling, through zinc sulfate, T1R3 or G-protein siRNA, blunted the protective effects of sucralose on the endothelium. Sucralose significantly reduced LPS-induced increased expression or phosphorylation of key signalling molecules, Src, PAK, MLC2, HSP27 and p110αPI3K.

Activation of T1R3, by sucralose, protects the pulmonary endothelium from edemagenic agent-induced barrier disruption, potentially through abrogation of Src/PAK/p110αPI3K-mediated cell-contact disassembly and Src/MLC2/HSP27-mediated actin-remodelling. Identification of sweet taste sensing in the pulmonary vasculature may represent a novel therapeutic target to protect the endothelium in settings of ARDS.

INTRODUCTION

Acute respiratory distress syndrome (ARDS) is a major cause of morbidity and mortality in patients suffering from several predisposing factors such as trauma, sepsis and pneumonia. The syndrome occurs when vascular fluid and protein leak across the pulmonary microvascular endothelium into the alveolar air space, causing pulmonary edema formation which is characteristic of the disease. Respiratory failure then occurs as a result of decreased gas exchange and lung compliance, and initiation of inflammatory cascades (79). Thus a key hallmark of ARDS is permeability of the pulmonary microvascular endothelium to vascular fluid and protein.

Vascular permeability is regulated through several mechanisms depending on the stimulus however each mechanism results in breakdown of cell-cell contacts and actin remodelling. Permeability of the monolayer occurs through disruption of cell-cell contacts, maintained by the adherens junction complex, and an increase in actin-myosin contractility (39, 40, 77). In the case of lipopolysaccharide (LPS), an endotoxin from gram-negative bacteria, endothelial permeability is mediated through its binding to toll-like receptor 4. The resulting Src-dependent signalling cascade leads to phosphorylation of both VE-cadherin and myosin light chain-2 (MLC2) (65, 71). Furthermore, expression of the heat shock protein families, HSP27, HSP 70 and HSP 90, correlates with increased vascular permeability (5, 32, 36). Targeting of these molecular mechanisms has been shown to attenuate LPS-induced pulmonary edema formation *in vivo* (3, 15), indicating the potential role for these molecules in settings of ARDS.

Members of the bitter taste receptor family, and their signalling effectors, have been identified in pulmonary solitary chemosensory cells (SCCs) (24, 37, 62, 67, 76), ciliated epithelial cells (64), and smooth muscle cells lining the airways (20). In pulmonary smooth muscle, 21 of the 25 members of the bitter taste receptor family have been identified with bitter taste agonists leading to vasodilation and bronchodilation (10, 20). While studies have identified other members of the taste receptor family in solitary chemosensory cells (SCCs), no functional output has been previously described (74). In recent years, sweet taste receptors have also been identified in extraoral locations, such as pancreatic beta cells, adipocytes and cardiomyocytes (6), however they have not been previously identified in the vasculature. Sweet taste is mediated by the G-protein coupled receptor (GPCR), T1R3, which can form a homodimer or a heterodimer with T1R2 (56). Sweet taste receptors are activated

upon binding of intensely sweet molecules, such as artificial sweeteners, at low concentrations (<1 mM) or glucose at high concentrations (>300 mM) (44). The consumption of artificial sweeteners has increased in recent years, with the concentration in diet soda ranging from 150 to 500 μM (25). In humans, while the majority of artificial sweeteners consumed are excreted in faeces, a significant proportion are absorbed by the small intestine, identified within the circulation (plasma) and excreted in the urine as a non-metabolized molecule (60, 72). Therefore it is likely that, following consumption of a diet high in artificial sweeteners, the vasculature is exposed to high levels of these intensely sweet molecules.

In the studies presented here, we demonstrate, for the first time, the presence of the sweet taste receptor T1R3 in the pulmonary endothelium. Expression of the receptor was demonstrated to be modulated by barrier-disruptive agents however stimulation of T1R3, with the intensely sweet artificial molecule sucralose, attenuates thrombin- and LPS-induced endothelial monolayer permeability. Furthermore, i*n vivo* exposure to sucralose attenuates lung edema formation induced by *Pseudomonas aeruginosa.* Our studies show that sucralose-mediated protection of the endothelial barrier is dependent on components of the sweet taste sensing pathway. Interestingly, exposure to high glucose does not protect the pulmonary endothelium. Finally, we implicate a role for HSP27, p110αPI3K, MLC2, Src and PAK in sucralose-mediated protection of the pulmonary endothelium. Our studies demonstrate that sweet taste sensing at the pulmonary endothelium plays a key role in barrier function. Stimulation of the sweet taste receptor may represent a novel target in the treatment of ARDS.

METHODS

Cell lines and reagents

TRIzol and Superscript II (Invitrogen). Rat lung microvascular endothelial cells (LMVEC; Vec Technologies, Rensselaer, NY) were cultured in MCDB-131 media (Vec Technologies) and used between *passages 3* and *9*. LPS (endotoxin) from *Escherichia coli* serotype 011:B4, recombinant VEGF protein and thrombin were purchased from Sigma-Aldrich (St. Louis, MO, USA). *Pseudomonas aeruginosa* strain 103 (PA103) was a kind gift from Dr. Troy Stevens (University of South Alabama, Mobile, AL, USA). Gustducin (*GNAT3*) and gustducin siRNA were purchased from Origene (Rockville, MD, USA). T1R3 (*Tas1R3*) and Gαq siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

In vivo studies

LPS or vehicle (saline) was administered to non-anesthetized, adult male 8- to 10-wk-old C57BL/6 mice via a single injection at different doses (1, 2.5 and 5 mg/kg i.p.). At 24 h following intraperitoneal injection of LPS or vehicle into mice, lungs were removed for homogenization. Untreated male Sprague-Dawley rats were euthanized at 8 weeks and both lungs and jejunal segments was isolated and stored in RNAlater (Thermo Scientific, Waltham, MA, USA) at −80 °C.

Mice were exposed to sucralose (1 g/kg) by oral gavage once a day for 1 week. At end-point, live gram-negative bacteria *P. aeruginosa* (PA103) or PBS vehicle was administered via a single intratracheal injection [10^6 colony-forming units (CFUs)]. At 4 h following PA103 administration, wet and dry lung weights were taken.

All animal experimental protocols were approved by the Institutional Animal Care and Use Committees of the Providence Veterans Affairs Medical Center and Brown University and comply with the Health Research Extension Act and U.S. Public Health Service policy.

RT-PCR

Total RNAs were extracted from rat lung, jejunum and cultured LMVECs using the TRIzol reagent (Thermo Scientific, Waltham, MA, USA) as per the manufacturer's instructions. RNA was purified using the acid phenol/chloroform system and reverse transcribed using SuperScriptII (Thermo Scientific, Waltham, MA, USA) and T1R3 transcripts were measured

with β-actin (GenBank accession number NM_031 144; forward 937-955, reverse 1223-1208) used as the house-keeping gene as described previously (8). Expression of the *Tas1r3* gene was measured using specific intron-spanning primers which were designed from the sequences published for rat (GenBank accession number NM_130818.1; forward 2107-2126, reverse 2327-2308). Relative gene expression level was analysed, for each sample, using the ΔCt method where $\Delta Ct = (Ct_{Tas1r3} - Ct_{\beta\text{-actin}})$ corresponding to the detected threshold cycles for the target gene and β-actin control.

Western blotting

LMVEC were exposed to LPS (1 μg/ml) or sucralose (0.1 mM) for 24 hours. Cells were then lysed with RIPA buffer, resuspended in Laemmli buffer and subjected to immunoblot analysis. Individual lobes of mouse lungs were homogenized in buffer [20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH7.9), 1.5 mM NaCl, 0.25 M sucrose, 0.2 mM EDTA, 200 mM PMSF, 0.5 mM DTT, and 1.5 mM $MgCl_2$] for 2 min and subjected to immunoblot analysis. Immunoblot analyses were performed on 10% SDS-PAGEs using a range of primary antibodies (table 1) at a dilution of 1:1000, except vinculin (1:5000), and secondary antibody dilutions of 1:5000. All samples were run on the same immunoblot for each protein analysed. Antibody specificity verification was assessed based on previous publications (included in table 1) or with siRNA knockout studies (Figure 5).

Endothelial monolayer permeability

Changes in endothelial monolayer permeability were assessed using the electrical cell impedance sensor (ECIS) technique (Applied Biophysics, Troy, NY), as previously described (16, 29). For analysis of monolayer permeability LMVEC were seeded to confluence onto collagen-coated electric cell-substrate impedance sensing arrays. For knockdown experiments, LMVECs were transiently transfected with T1R3, Gαq or gustducin siRNA duplexes (300 nM), or ns, scrambled control, using the Amaxa (Allendale, NJ, USA) electroporation technique as described previously (15). Monolayers were treated with either sucralose (0.1 mM), glucose (5.5 mM, 11 and 25 mM) or vehicle (H_2O) in the presence and absence of VEGF (50 ng/ml), thrombin (2 U/ml), LPS (1 μg/ml) or zinc sulfate (0.7 mM). Addition of treatments were made at the same time and resistance was measured over time.

Statistical analysis

For three or more groups, differences among the means were tested for significance in all experiments by ANOVA with Fisher's least significance difference test. Significance was reached when $p < 0.05$. Values are mean ± standard deviation (SD).

RESULTS

Sweet taste receptor, T1R3, is expressed at the pulmonary endothelium

Sweet taste receptor T1R3 is the key component of the sweet taste complex – T1R3 is necessary for the heterodimeric complex but can also form a homodimer for sweet taste sensing (19, 50, 56). In addition to the oral cavity, high expression of T1R3 mRNA (*TAS1R3*) and protein has been found in the small intestine, in particular the jejunum (41). mRNA expression levels of *TAS1R3* in rat lungs and LMVEC were comparable to the positive control rat tissue (jejunum) (Figure 1a). To assess the link between sweet taste receptor and ARDS, protein expression of T1R3 was studied in LMVEC following exposure (24 h) to the barrier disruptive agents LPS, VEGF and thrombin, and in mouse lungs following exposure to LPS (4 h). In LMVEC, T1R3 protein levels were significantly reduced, to a similar degree, in the presence of all three agonists (Figure 1b). Expression of T1R3 in mouse lungs was unaffected at low concentrations of LPS (1 and 2.5 mg/kg) however at 5 mg/kg, where lung injury and vascular leak are observed (13, 15), T1R3 expression was significantly reduced (Figure 1c). This data demonstrates the presence of T1R3 in the lung microvasculature, and implicates sweet taste sensing in endothelial barrier function.

Artificial sweetener sucralose attenuates barrier disruption in vitro and in vivo

The sweet taste receptor complex is activated by low concentrations of intensely sweet molecules or high concentrations of sugars (51). Our previous studies demonstrate that endothelial permeability is closely related to adherens junction formation, with increased VE-cadherin surface levels observed in barrier protective settings (15). Therefore we next assessed whether activation of T1R3 with the artificial sweetener sucralose, at concentration close to EC_{50} (51), has an effect on endothelial barrier function and VE-cadherin surface expression. LMVEC exposed to sucralose displayed no change in endothelial monolayer resistance (Figure 2a, 2b, 3a and 3b) or VE-cadherin surface expression (Figure 2c and 3c). Interestingly, thrombin-induced permeability and loss of VE-cadherin surface expression was significantly attenuated by concomitant exposure of LMVEC to sucralose (Figure 2). Likewise, sucralose attenuated LPS-induced permeability and decrease in VE-cadherin surface levels (Figure 3). We next sought to establish whether sucralose exerted a protective effect on *in vivo* lung edema formation (wet-to-dry lung weight). Mice were exposed to a daily oral dose of sucralose over a 1 week period, followed by exposure to *P. aeruginosa* (PA103) as a model

for acute lung injury. Similar to *in vitro* findings, sucralose exposure significantly attenuated PA103-induced lung edema formation *in vivo* (Figure 3d). Interestingly, sucralose exposure in the absence of PA103 had no effect on lung edema formation. Interestingly, both LPS- and thrombin-induced permeability *in vitro* and PA103-induced edema formation *in vivo* was not completely reversed by sucralose however the artificial sweetener did result in surface expression levels of VE-cadherin returning to baseline levels (Figure 2 and 3).

We next assessed whether glucose regulates endothelial barrier function in a similar manner. LMVEC were exposed to increasing concentrations of glucose from fasting levels (5.5 mM) to hyperglycaemic levels (25 mM), with an osmotic control of mannose used for the high glucose concentration, in the presence and absence of LPS. High glucose (25 mM), but not lower glucose concentrations or mannose, significantly increased endothelial permeability and decreased VE-cadherin surface levels under baseline conditions (Figure 4a and b). LPS-induced permeability and decreased VE-cadherin surface levels was significantly exacerbated in the presence of high glucose, but not lower glucose concentrations or mannose (Figure 4a and b). Interestingly, exposure of LMVEC to sucralose significantly increased protein levels of T1R3, whilst high glucose had no effect on expression of the sweet taste receptor (Figure 4c).

Taken together, these data indicate that the intensely sweet molecule sucralose, but not high physiological levels of glucose, regulates T1R3 to protect the pulmonary endothelium against barrier disruption.

Barrier-protective effect of sucralose is mediated through sensing by the sweet taste receptor

To study whether sucralose acts on the endothelial monolayer in a T1R3-dependent manner, the next experiments utilised inhibitors of the sweet taste receptor pathway. Molecular and chemical inhibition of T1R3 was performed using siRNA knockdown (Figure 5a) and exposure to zinc sulfate (Figure 5b) a chemical inhibitor of sweet taste receptor (23, 38). Endothelial permeability was assessed in the presence and absence of LPS and sucralose. Interestingly, attenuation of LPS-induced permeability by sucralose was significantly blocked by molecular (Figure 5aii) and chemical (Figure 5b) inhibition of T1R3. In the presence of LPS alone, T1R3 inhibition had no impact on endothelial permeability (Figure 5a and b). Molecular inhibition of gustducin, a key signalling molecule downstream of T1R3 (53), was performed

using siRNA knockdown (Figure 5ci). Knockdown of gustducin, had no effect on endothelial permeability in settings of either LPS or sucralose exposure (Figure 5cii). Molecular inhibition of gustducin significantly abrogated sucralose-mediated protection of LPS-induced permeability (Figure 5cii). The G protein, Gαq, which is highly expressed in the lung has also been identified to play a role in sweet taste sensing (75, 80). Molecular inhibition of Gαq was performed in LMVEC using siRNA (Figure 5di). Protection of LPS-induced permeability, by sucralose, was reduced by 21% following knockdown of Gαq (Figure 5d). These data indicate that sucralose exerts a protective effect on the endothelium, in settings of barrier disruption, through regulation of the sweet taste receptor and the downstream signalling pathway.

Sucralose attenuates LPS-induced elevated HSP27 and p110α and activation of MLC2, Src and PAK

To assess the molecular mechanism through which sucralose exerts an effect on LPS-induced signalling, key regulators of the adherens junction and endothelial barrier function were assessed for expression and activity. Phosphorylation of kinases FAK, p38, ERK, PAK, p70 and Src (15, 28), phosphatase SHP2 (14), filament proteins VASP and cofilin (59, 67), and MLC2 (7) were measured at phosphorylation sites relevant to protein activity (Table 1). Expression of heat shock proteins HSP27, 70 and 90 (11, 36, 45) and the p110αPI3K (9) were also assessed. Sucralose treatment, in the absence of LPS, had no effect on phosphorylation or expression of any regulator molecule (Figure 6 and 7). Phosphorylation of MLC2, Src and PAK by LPS was significantly attenuated by exposure to sucralose (Figure 6 a, 6b and 6c), whereas phosphorylation of other key regulators was unaffected by sucralose (Figure 7a-g). Unlike MLC2 and Src, in the presence of sucralose and LPS, phosphorylation of PAK did not return to baseline conditions (Figure 6a, 6b and 6c). Expression levels of HSP27 and p110αPI3K were increased following exposure to LPS however this effect was abrogated by sucralose (Figure 6d and 6e). This effect was not observed in the other heat shock proteins, HSP70 and 90 (Figure 7h and 7i). Taken together, these data indicate that sucralose may attenuate LPS-induced permeability through inhibition of key barrier disruptive signaling molecules.

DISCUSSION

In the present study we demonstrate, for the first time, the localisation and function of the sweet taste receptor at the pulmonary endothelium. Our research identified the expression of T1R3 in the lung and microvascular endothelial cells, with reduced protein levels in response to the barrier-disruptive agents LPS, thrombin and VEGF. We observed that activation of T1R3, by exposure to the artificial sweetener sucralose, protects the microvasculature *in vitro* and *in vivo* against barrier disruptive agents, through a sweet taste receptor-dependent pathway. Lastly, we implicated a role for sucralose in attenuating LPS-mediated Src, PAK, MLC2, HSP27 and p110αPI3K signalling. Therefore the stimulation of T1R3, by artificial sweetener sucralose, represents a novel mechanism through which the pulmonary microvasculature is regulated.

The sweet taste receptor, T1R3, was first identified at the *Sac* genetic locus, which regulates sweet taste sensitivity, with expression observed in a subset of taste cells within the oral cavity (52, 54). Interestingly, T1R3 has recently been identified in extraoral locale, including the pancreatic beta cell, adipocytes and the bladder, however to date, no studies have assessed T1R3 in the vasculature (23, 55, 66, 73). Our study identifies T1R3 mRNA expression in the rat lung and microvascular endothelial cells. Interestingly, mRNA levels of both were similar to those observed in the jejunum segment of the small intestine. Studies by others and us have identified T1R3 in different cell types within the small intestine, predominantly the jejunum, where activation of the receptor is linked to altered glucose metabolism in patients with metabolic diseases (8, 47, 49, 69). Therefore T1R3 expression in the pulmonary vasculature is likely to be at a physiologically significant level. Endothelial cell protein expression of T1R3 was reduced by the barrier disruptive agents LPS, thrombin and VEGF. This was mirrored in the mouse lung where decreased T1R3 levels were noted following LPS treatment. Interestingly, at low doses of LPS, where no pulmonary edema is observed (data not shown), T1R3 expression is not significantly affected however at 5 mg/kg dose, where pulmonary edema is observed (13, 15), T1R3 expression was significantly reduced. Therefore it is likely that T1R3 expression plays a role in pulmonary endothelial barrier maintenance *in vivo* and *in vitro.* Indeed, following exposure to sucralose, which activates T1R3, barrier permeability caused by LPS and thrombin was attenuated. These findings were mirrored in an *in vivo* model of lung injury (*P. aeruginosa)*, with sucralose exposure blocking lung edema formation. The *in vitro* protective role of sucralose was blocked

following inhibition of sweet taste sensing, either by acting on the receptor via zinc sulfate (23, 38) or siRNA knockdown of T1R3 or downstream G proteins gustducin and Gαq (53, 75). Inhibition of T1R3, through siRNA or zinc sulfate, attenuated the protective effect of sucralose on the endothelial barrier. The artificial sweetener is therefore acting through the sweet taste receptor to initiate a protective signalling response. Interestingly, Gαq inhibition did not completely blunt sucralose-mediated protection as seen in gustducin inhibition. It is therefore likely that gustducin, but not Gαq, is essential for T1R3-mediated signalling in the pulmonary endothelium.

Signalling mechanisms mediated by activated T1R3 vary depending on the cell type. In the pancreatic beta cell, sweetener-T1R3 binding results in insulin release mediated by elevated intracellular calcium levels (55) whilst in the adipocyte, Akt phosphorylation was noted to play a role in the stimulation of adipogenesis (66). In the pulmonary endothelium, studies presented here link the phosphorylation of Src, PAK and MLC2, and increased expression of HSP27 and p110αPI3K, with sucralose-mediated protection from LPS. Previous studies have indicated a key role for Src and PAK phosphorylation, and p110αPI3K expression, in the breakdown of the pulmonary endothelium through dissolution of the adherens junction (9, 12, 15, 28) and in HSP27 and MLC2-mediated actin remodelling associated with barrier disruption (27, 32, 65, 71). Interestingly, other key regulators of the pulmonary endothelium, such as the filament proteins cofillin and VASP (59, 67) are phosphorylated by LPS but unaffected by sucralose. Thus, upon activation, T1R3 acts on a limited range of signalling molecules to regulate endothelial barrier function. The link between signalling downstream of T1R3 and Src/PAK/p110αPI3K and HSP27/MLC2 is unclear at present however it is possible that PLCβ2 recruitment, following release of gustducin and Gαq, triggers the activation of kinases such as the inhibitory Src kinase Csk (48, 81). This in turn may regulate downstream molecules to protect the endothelial barrier from LPS-induced disruption. However further studies are necessary to identify and understand the molecular mechanisms through which T1R3 downstream signalling regulates Src/PAK/p110αPI3K and HSP27/MLC2 within the pulmonary endothelium.

Sucralose is an intensely sweet, commercially-available artificial sweetener with an estimated ‘sweetness’ index of 600-times compared to sucrose (57). Sucralose, like many artificial sweeteners, stimulates the sweet taste receptor at low concentrations (<1 mM) (44). At glucose concentrations needed to stimulate T1R3 (>300 mM), endothelial cells are not

viable due to hyperosmolarity (1, 21). We demonstrate that, at a physiologically relevant high concentration of glucose (25 mM), vascular permeability was increased. Similar to previous studies, we also observed that high glucose exacerbates LPS-mediated barrier disruption (46) therefore the protective effect of T1R3 activation, with sucralose, cannot be mimicked by glucose. Furthermore, whilst this study focused on the use of sucralose to activate T1R3, different artificial sweeteners demonstrate varying ability to bind T1R3 and stimulate downstream signalling (56). It is therefore possible that the level of pulmonary barrier protection exhibited by the sweetener is dependent on the type of sweet molecule used.

There is significant controversy regarding the benefit of artificial sweetener consumption in the diet. At present, a large proportion of the population consumes artificial sweeteners, such as sucralose, at high levels (25) however clinical studies do not record any pulmonary responses in this population. Interestingly, our studies show that exposure of the microvasculature to sucralose, in the absence of LPS, has no effect on barrier function or on the expression or activation of key signalling molecules which regulate the endothelium. Therefore it is possible that stimulation of T1R3, by artificial sweeteners, only plays a physiological role in settings of vascular permeability. This represents the potential for artificial sweeteners to act as a novel therapeutic agent in diseases such as ARDS, however further studies are necessary to assess the long-term effect of artificial sweeteners on the pulmonary vasculature. Whilst the present study only assessed T1R3 expression, as it is the predominant sweet taste receptor which homodimerises to sense sweet molecules, T1R2 can form a heterodimer with T1R3 and form a sweet taste receptor complex (56). Furthermore, T1R3 can heterodimerise with T1R1 to form an umami taste receptor complex. As our study demonstrates a significant protective effect played by the sweet taste receptor in the pulmonary endothelium, it would be interesting to assess other taste sensing complexes within the vasculature. In fact, previous studies have implicated that stimulation of the bitter taste receptor family (T2R) in airway smooth muscle and epithelial cells, with bitter taste agonists, stimulates bronchodilation and ciliary beat frequency respectively (20, 64). Bitter agonists are currently under scrutiny as a treatment for asthma and COPD patients (42, 61, 68) however studies are yet to assess the presence or activation of bitter taste receptors within the pulmonary vasculature. Our studies demonstrate that sweet taste agonists block the barrier disruptive effects of LPS on the pulmonary endothelium. There is therefore the potential for taste agonists to play a major role in various lung diseases in the future.

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AUTHOR CONTRIBUTIONS

Acquisition of data: HC, AV, JB, AS

Conception and design: HC, EOH

Analysis and interpretation: HC, AS, AV

Drafting the manuscript for intellectual content: HC, EOH

CONFLICT OF INTEREST DISCOSURE

The authors have nothing to disclose.

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FIGURE LEGENDS

Figure 1: Expression of the sweet taste receptor, T1R3, at the pulmonary endothelium is regulated by barrier disruptive agents. *Panel a:* mRNA expression of the T1R3 gene, *Tas1r3*, in rat lung and jejunum tissue and cultured rat LMVEC. Gene expression is relative to the housekeeping gene, β-actin, and normalized to the positive control jejunum tissue. n = 6. *Panel b and c:* Protein expression of T1R3 in: (b) cultured rat LMVEC exposed to LPS (1 μg/ml), thrombin (2 U/ml) or VEGF (50 ng/ml) for 24 hours and (c) homogenates of lungs from C57/BL6 mice exposed to varying doses of LPS (0 - 5 mg/kg). n = 5. A representative blot (i) and densitometry relative to the load control, β-actin (ii) are shown. Data is expressed as mean ± S.D. *p<0.05 versus vehicle.

Figure 2: Stimulation of the artificial sweetener sucralose attenuates thrombin-induced barrier disruption and VE-cadherin internalisation. *Panels a and b:* Changes in rat LMVEC endothelial monolayer resistance were measured using ECIS in the presence (closed symbols) and absence (open symbols) of thrombin (2 U/ml). Monolayers were exposed to sucralose (0.1 mM) (triangle symbols), or vehicle (H_2O) (square symbols) at the same time as thrombin. Permeability is shown as (a) an experimental trace, normalised to the addition of thrombin and sucralose (arrow), and (b) drop in endothelial resistance measured at 12 minutes post-thrombin and sucralose treatment. n = 5. *Panel c:* Cell surface expression of VE-cadherin was determined, with whole-cell indirect ELISA using chemiluminescence, following exposure to thrombin and sucralose as per (a). n = 6. Data is expressed as mean ± S.D. *p<0.05 versus vehicle for thrombin, $^{\#}$p<0.05 vs vehicle for sucralose.

Figure 3: Stimulation of the artificial sweetener sucralose attenuates LPS-induced barrier disruption and VE-cadherin internalisation *in vitro* and bacteria-induced edema formation *in vivo*. *Panels a and b:* Changes in rat LMVEC endothelial monolayer resistance were measured using ECIS in the presence (closed symbols) and absence (open symbols) of LPS (1 μg/ml). Monolayers were exposed to sucralose (0.1 mM) (triangle symbols), or vehicle (H_2O) (square symbols) at the same time as LPS. Permeability is shown as (a) an experimental trace, normalised to the addition of LPS and sucralose (arrow), and (b) drop in endothelial resistance measured at 10 hours. n = 5. *Panel c:* Cell surface expression of VE-cadherin was determined,

with whole-cell indirect ELISA using chemiluminescence, following exposure to LPS and sucralose as per (a). n = 6. *Panel d*: Lung edema formation was determined by measuring wet-to-dry lung weight ratio in mice following daily gavage of sucralose (1 g/kg) for 1 week and 4 hour exposure to *P. aeruginosa* (PA103). n=5-8. Data is expressed as mean ± S.D. *p<0.05 versus vehicle for LPS, $^{\#}$p<0.05 vs vehicle for sucralose.

Figure 4: High glucose exposure increases endothelial barrier permeability and VE-cadherin internalisation. *Panel a:* Changes in rat LMVEC endothelial monolayer resistance (*panel a)* were measured using ECIS in the presence (closed bars) and absence (open bars) of LPS (1 µg/ml). Monolayers were exposed to different concentrations of glucose (5.5, 11 and 25 mM) or osmotic control mannose (25 mM) at the same time as LPS. Permeability is shown as drop in endothelial resistance measured at 10 hours. n = 5. *Panel b:* Cell surface expression of VE-cadherin was determined, with whole-cell indirect ELISA using chemiluminescence, following exposure to LPS and glucose as per (a). *Panel c:* Protein expression of T1R3 in cultured rat LMVEC exposed to sucralose (0.1 mM), glucose (25 mM) or vehicle for both (H_2O) for 24 hours. A representative blot (*upper panel*) and densitometry relative to the load control, β-actin (*lower panel*) are shown. n = 5. Data is expressed as mean ± S.D. *p<0.05 versus vehicle for LPS, $^{\#}$p<0.05 versus 5.5 mM control.

Figure 5: Barrier-protective effect of sucralose is mediated through sensing by the sweet taste receptor. *Panels a, c and d:* Equivalent numbers of rat LMVECs were transiently transfected with scrambled (300 nM, open bars) or T1R3 (300 nM, closed bars) siRNA (*panel a ii)*, gustducin (Gus, 300 nM, closed bars) siRNA *(panel c ii)* or Gαq (300 nM, closed bars) siRNA (*panel d ii*). Following 48 hours, changes in endothelial monolayer resistance were measured using ECIS in the presence and absence of LPS (1 µg/ml) and sucralose (0.1 mM). Permeability is shown as drop in endothelial resistance measured at 10 hours (*ii*). Knockdown of endogenous protein was confirmed by immunoblot analysis of lysates from transiently transfected cells with an antibody specific to T1R3 (*panel a i*), gustducin (*panel c i*) and Gαq (*panel d i*). *Panel b:* Monolayer permeability was assessed in the presence and absence of the sweet taste inhibitor zinc sulfate (0.7 mM). Changes in endothelial monolayer resistance were measured using ECIS in the presence and absence of LPS (1 µg/ml) and sucralose (0.1 mM). Permeability is shown as drop in endothelial resistance measured at 10 hours. n = 5-6.

694 Data is expressed as mean ± S.D. *p<0.05 versus vehicle for LPS, ^p<0.05 vs vehicle for
695 sucralose, #p<0.05 versus LPS + vehicle for sucralose.
696
697 **Figure 6: Sucralose attenuates LPS-induced elevated HSP27 and p110αPI3K and activation**
698 **of MLC2, Src and PAK.** Rat LMVECs were treated in the presence or absence of LPS (1 μg/ml)
699 and sucralose (0.1 mM) for 24 hours. Phosphorylation of MLC-2 (a), Src (b) and PAK (c) was
700 assessed in whole-cell lysates by immunoblot analysis with an antibody specific to each
701 phosphorylated protein. Blots were stripped and reprobed for total protein expression and
702 actin as a loading control. Total protein expression of HSP27 (d) and p110αPI3K (e) was also
703 assessed in whole-cell lysates, followed strip and reprobe of blots for actin as a loading
704 control. Representative blots are shown. Non-essential lanes from the HSP27 representative
705 blot (panel d) have been removed. n = 6. Data is expressed as mean ± S.D. *p<0.05 versus
706 vehicle for LPS, #p<0.05 vs vehicle for sucralose.
707
708 **Figure 7: Role of sucralose on LPS-mediated signalling is independent of several key**
709 **molecules.** Rat LMVECs were treated in the presence or absence of LPS (1 μg/ml) and
710 sucralose (0.1 mM) for 24 hours. Phosphorylation of HSP70 (a), SHP2 (b), ERK (c), FAK (d),
711 VASP (e), p38 (f) and cofillin (g) was assessed in whole-cell lysates by immunoblot analysis
712 with an antibody specific to each phosphorylated protein. Blots were stripped and reprobed
713 for total protein expression and actin as a loading control. Total protein expression of HSP70
714 (h) and HSP90 (i) was also assessed in whole-cell lysates, followed strip and reprobe of blots
715 for actin as a loading control. Representative blots are shown. n = 6. Data is expressed as
716 mean ± S.D. *p<0.05 versus vehicle for LPS.
717
718

TABLE

Table 1: List of antibodies used for protein phosphorylation (panel a) and expression (panel b) analysis by Western blot.

a

Antibody	**Company**	**Phospho site**
Phospho-Cofilin	Cell Signaling (30)	Serine 3
Phospho-MLC2	Cell Signaling (22)	Threonine 18 / serine 19
Phospho-VASP	Cell Signaling (31)	Serine 239
Phospho-PAK 1/2	Cell Signaling (33)	Threonine 423/402
Phospho-Src	Cell Signaling (4)	Tyrosine 416
Phospho-ERK1/2	Cell Signaling (70)	Threonine 202 / tyrosine 204
Phospho-p38	Cell Signaling (17)	Threonine 180 / tyrosine 182
Phospho-p70 (T389)	Cell Signaling (78)	Threonine 389
Phospho-FAK (Y397)	Cell Signaling (12)	Tyrosine 397
Phospho-SHP2 (Y542)	Santa Cruz (12)	Tyrosine 452

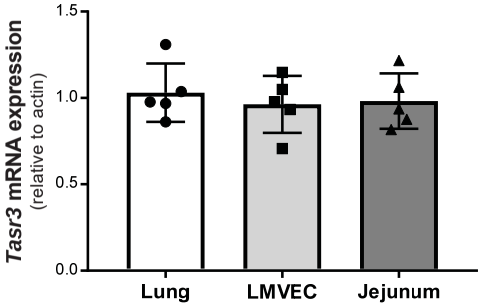
b

Antibody	**Company**
HSP90	BD Biosciences (43)
FAK	BD Bioscience (12)
HSP70	BD Biosciences (18)
Cofilin	Cell Signaling (58)
VASP	Cell Signaling (31)

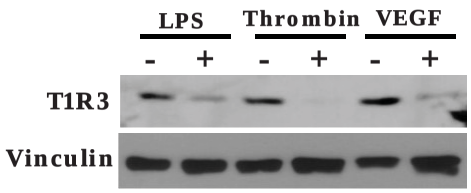
PAK1	Cell Signaling (4)
MLC2	Cell Signaling (22)
ERK1/2	Cell Signaling (35)
p38	Cell Signaling (17)
Src	Santa Cruz (34)
SHP2	Santa Cruz (12)
p70	Santa Cruz (26)
T1R3	Santa Cruz
β-actin	Santa Cruz (2)
Gαq	Santa Cruz
p110αPI3K	Santa Cruz (82)
Gustducin	Santa Cruz
HSP27	Santa Cruz (63)

Figure 1

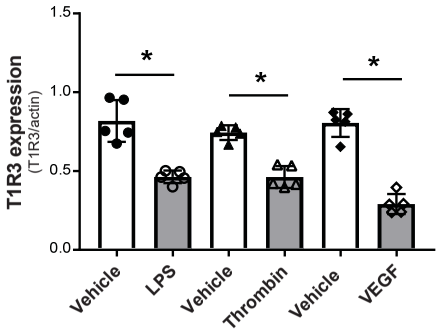
a



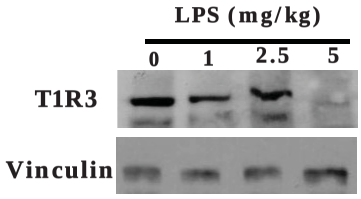
b i)



b ii)



c i)



c ii)

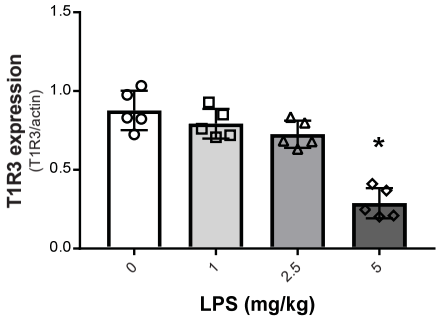
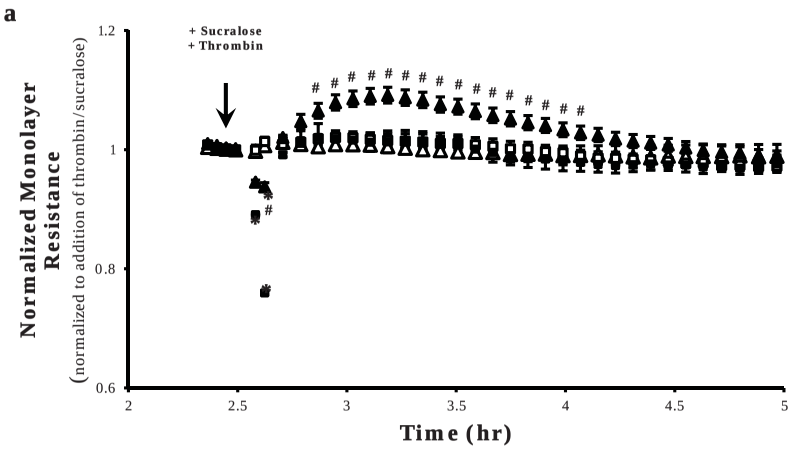
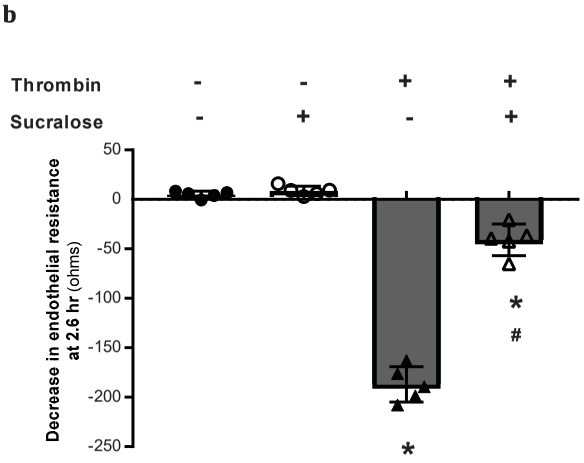


Figure 2


a
+ Sucralose
+ Thrombin
Normalized Monolayer Resistance
(normalized to addition of thrombin/sucralose)
1.2
1
0.8
0.6
2
2.5
3
3.5
4
4.5
5
Time (hr)


b
Thrombin - - + +
Sucralose - + - +
Decrease in endothelial resistance at 2.6 hr (ohms)
50
0
-50
-100
-150
-200
-250

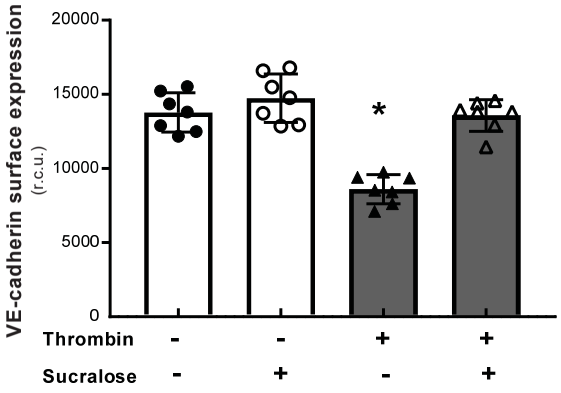

c
VE-cadherin surface expression
(r.c.u.)
20000
15000
10000
5000
0
Thrombin - - + +
Sucralose - + - +

Figure 3

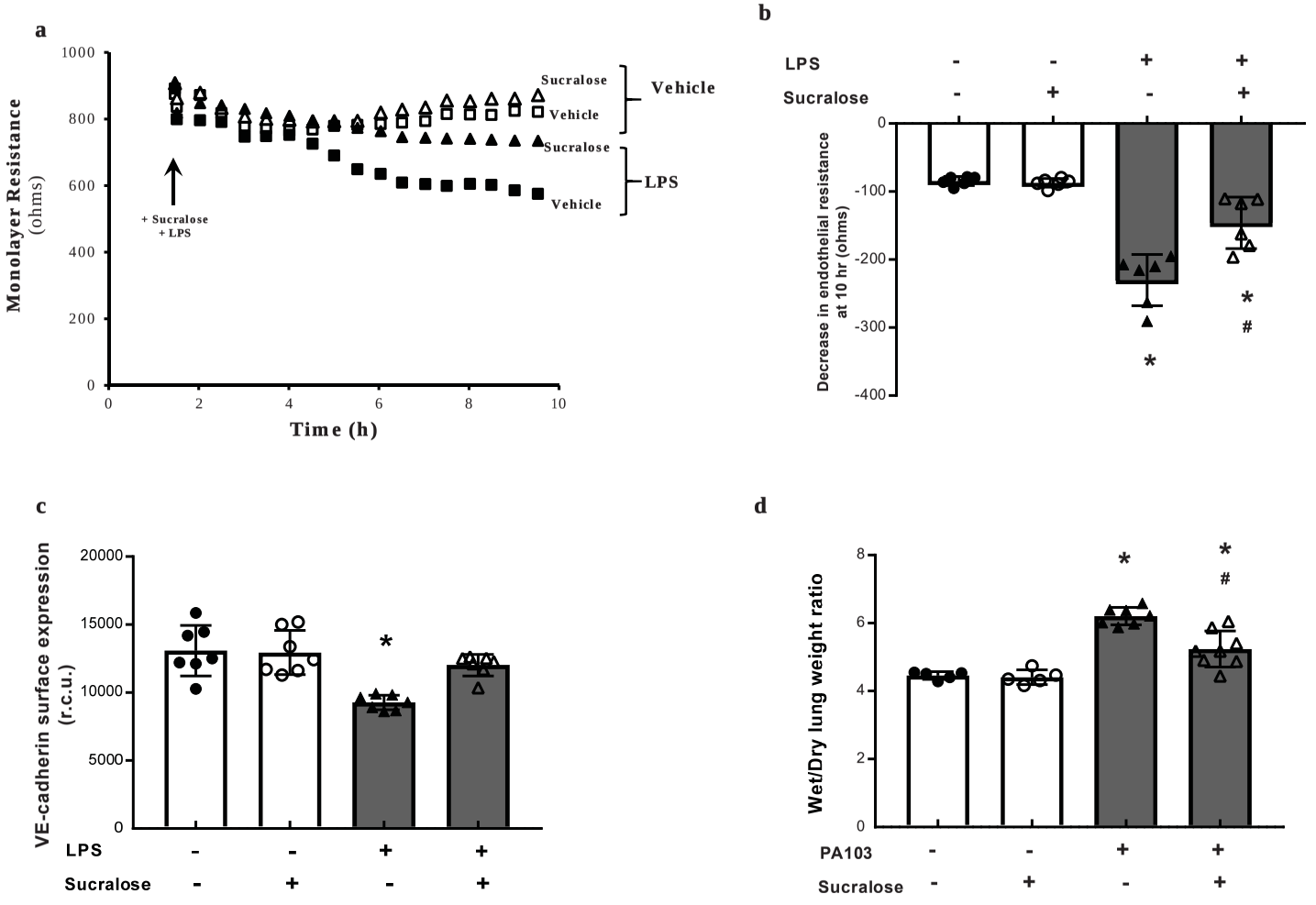

a
Monolayer Resistance (ohms)
1000
800
600
400
200
0
0
2
4
6
8
10
Time (h)
+ Sucralose
+ LPS
Sucralose
Vehicle
Vehicle
Sucralose
Vehicle
LPS
b
LPS
Sucralose
Decrease in endothelial resistance at 10 hr (ohms)
0
-100
-200
-300
-400
c
VE-cadherin surface expression (r.c.u.)
20000
15000
10000
5000
0
LPS
Sucralose
d
Wet/Dry lung weight ratio
8
6
4
2
0
PA103
Sucralose

Figure 4

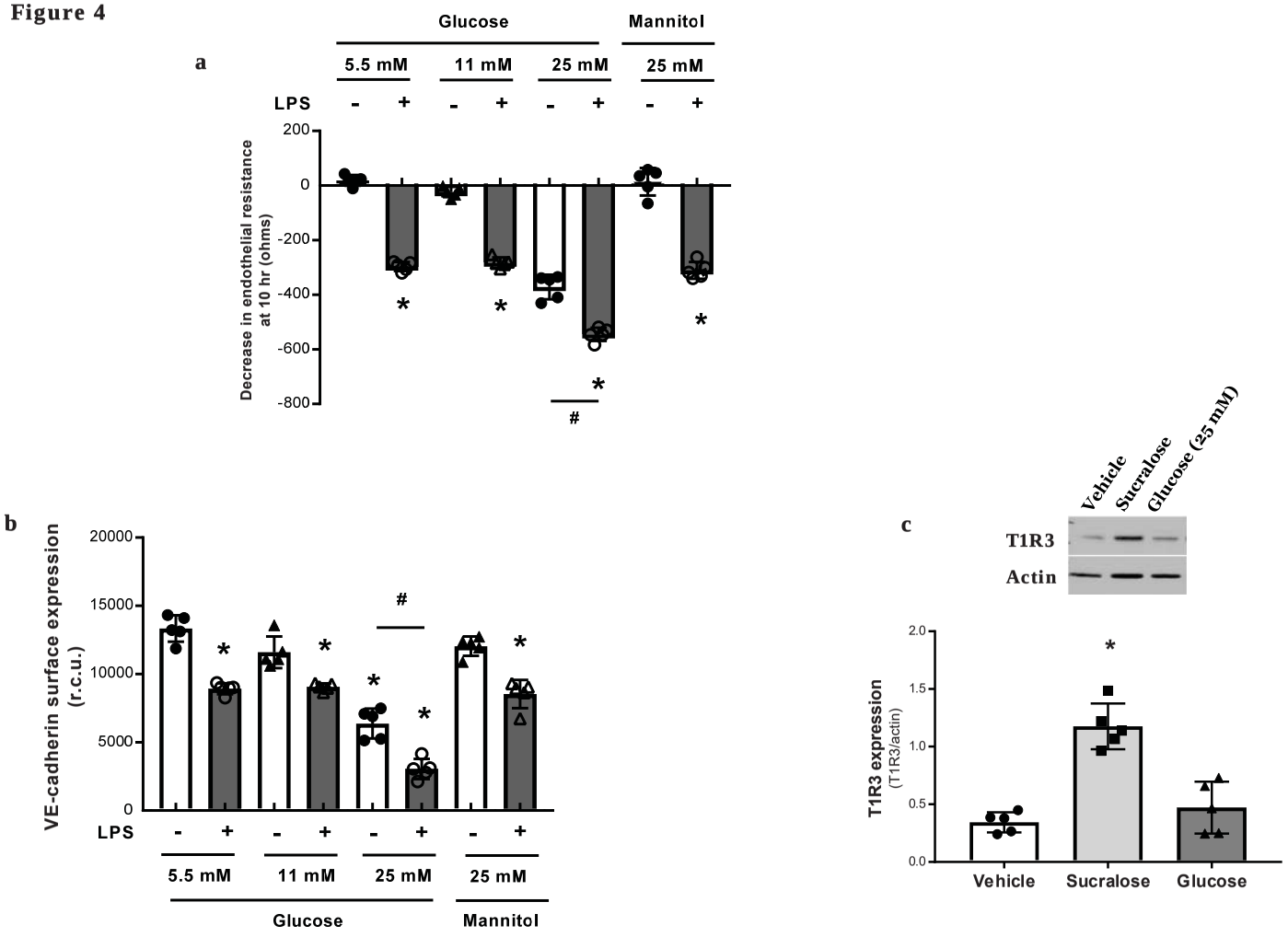

a
Glucose
Mannitol
5.5 mM
11 mM
25 mM
25 mM
LPS
Decrease in endothelial resistance at 10 hr (ohms)
b
VE-cadherin surface expression (r.c.u.)
c
Vehicle
Sucralose
Glucose (25 mM)
T1R3
Actin
T1R3 expression (T1R3/actin)

Figure 5

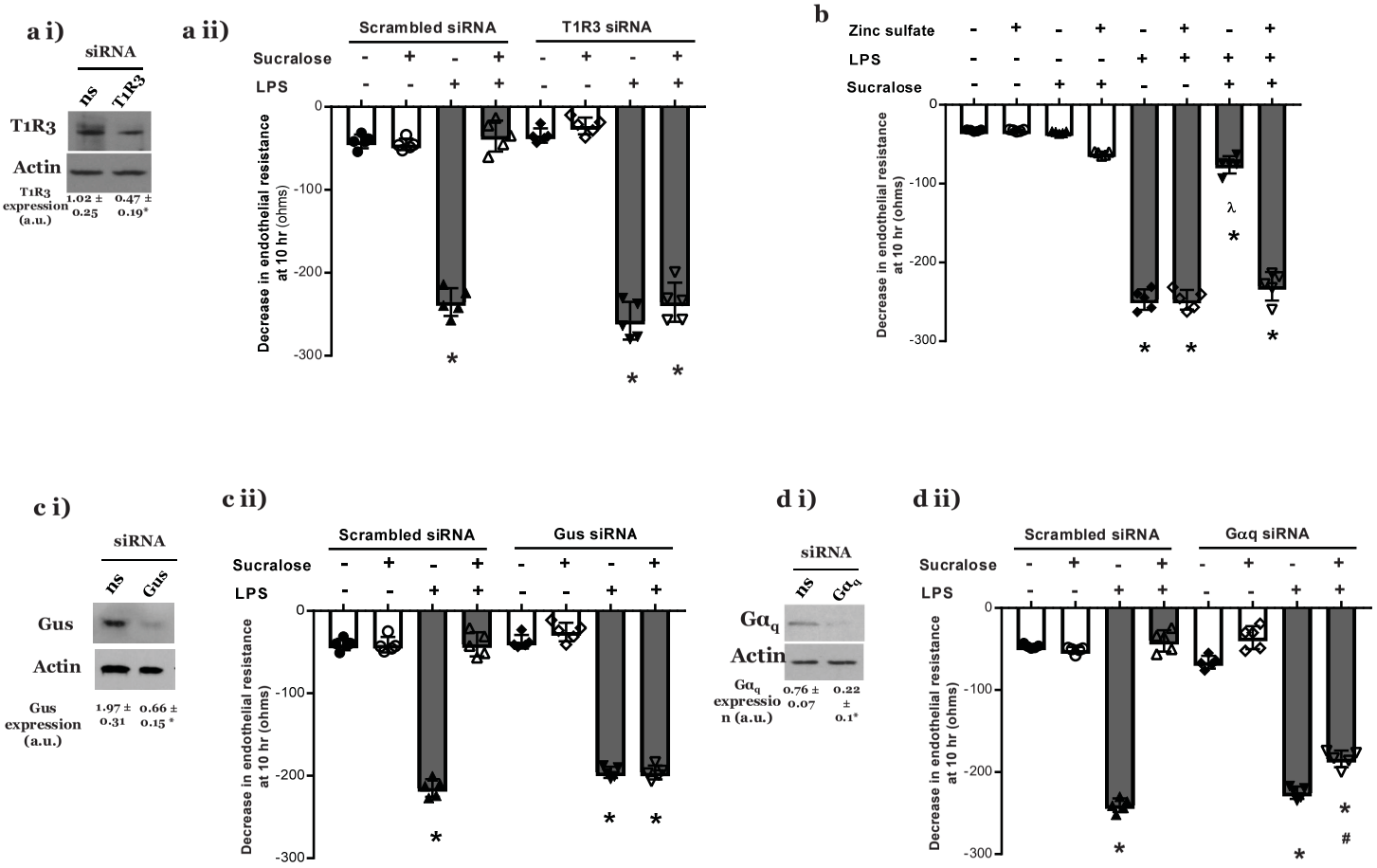

a i)
siRNA
ns
T1R3
T1R3
Actin
T1R3 expression (a.u.)
1.02 ± 0.25
0.47 ± 0.19*
a ii)
Scrambled siRNA
T1R3 siRNA
Sucralose
LPS
Decrease in endothelial resistance at 10 hr (ohms)
0
-100
-200
-300
b
Zinc sulfate
LPS
Sucralose
Decrease in endothelial resistance at 10 hr (ohms)
λ
c i)
siRNA
ns
Gus
Gus
Actin
Gus expression (a.u.)
1.97 ± 0.31
0.66 ± 0.15 *
c ii)
Scrambled siRNA
Gus siRNA
Sucralose
LPS
Decrease in endothelial resistance at 10 hr (ohms)
d i)
siRNA
ns
Gαq
Gαq
Actin
Gαq expression (a.u.)
0.76 ± 0.07
0.22 ± 0.1*
d ii)
Scrambled siRNA
Gαq siRNA
Sucralose
LPS
Decrease in endothelial resistance at 10 hr (ohms)
#

Figure 6

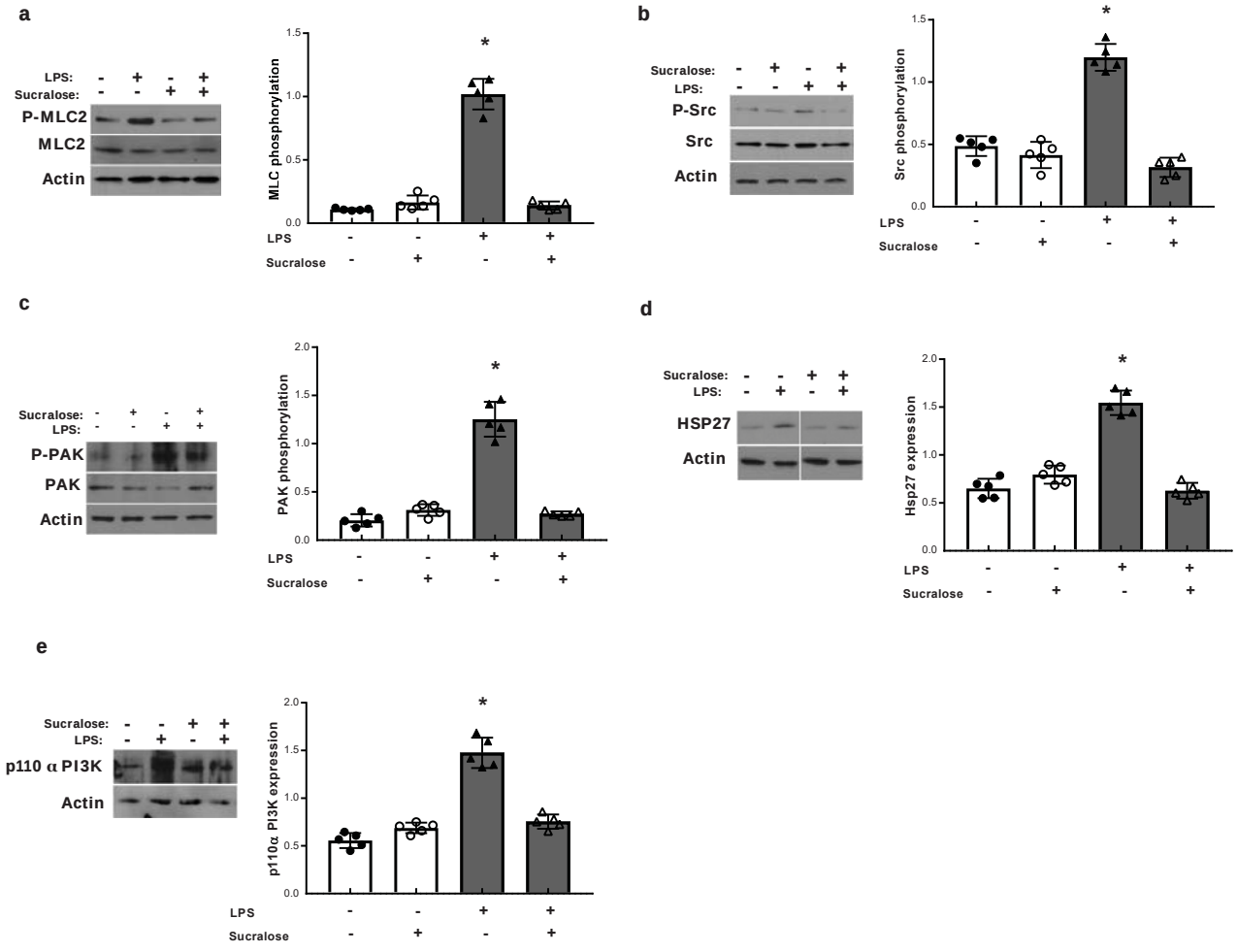
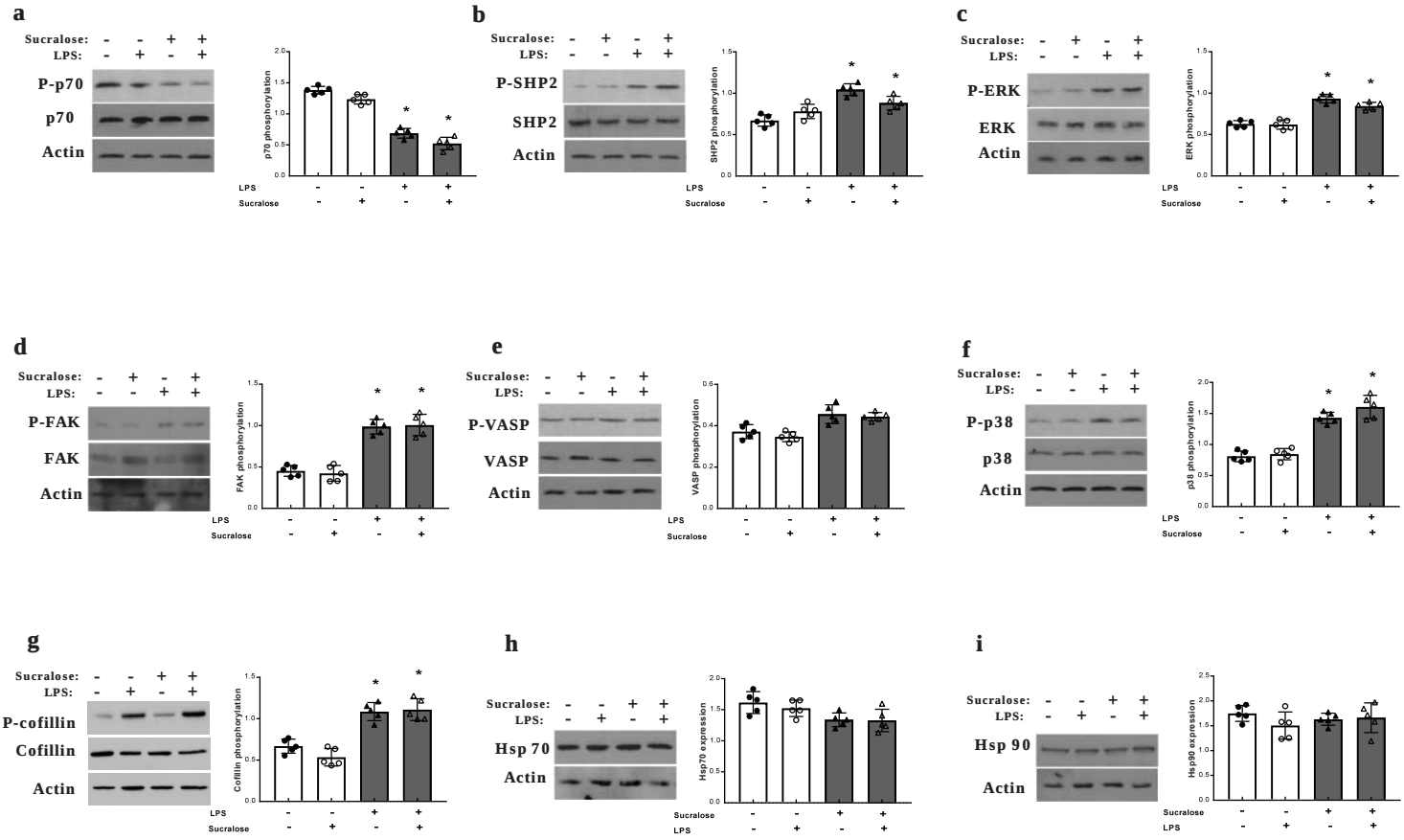

a
LPS:
Sucralose:
P-MLC2
MLC2
Actin
MLC phosphorylation
b
P-Src
Src
Actin
Src phosphorylation
c
P-PAK
PAK
Actin
PAK phosphorylation
d
HSP27
Actin
Hsp27 expression
e
p110 α PI3K
Actin
p110α PI3K expression

Figure 7


a
Sucralose: - - + +
LPS: - + - +
P-p70
p70
Actin
p70 phosphorylation
LPS - - + +
Sucralose - + - +
b
Sucralose: - + - +
LPS: - - + +
P-SHP2
SHP2
Actin
SHP2 phosphorylation
LPS - - + +
Sucralose - + - +
c
Sucralose: - + - +
LPS: - - + +
P-ERK
ERK
Actin
ERK phosphorylation
LPS - - + +
Sucralose - + - +
d
Sucralose: - + - +
LPS: - - + +
P-FAK
FAK
Actin
FAK phosphorylation
LPS - - + +
Sucralose - + - +
e
Sucralose: - + - +
LPS: - - + +
P-VASP
VASP
Actin
VASP phosphorylation
LPS - - + +
Sucralose - + - +
f
Sucralose: - + - +
LPS: - - + +
P-p38
p38
Actin
p38 phosphorylation
LPS - - + +
Sucralose - + - +
g
Sucralose: - - + +
LPS: - + - +
P-cofillin
Cofillin
Actin
Cofillin phosphorylation
LPS - - + +
Sucralose - + - +
h
Sucralose: - - + +
LPS: - + - +
Hsp 70
Actin
Hsp70 expression
Sucralose - - + +
LPS - + - +
i
Sucralose: - - + +
LPS: - + - +
Hsp 90
Actin
Hsp90 expression
Sucralose - - + +
LPS - + - +